

AD_____

Award Number: DAMD17-01-1-0247

TITLE: Downstream Signaling Mechanism Underlying MAPK-Induced
Generation of the ER-Negative Phenotype

PRINCIPAL INVESTIGATOR: Jamie N. Holloway

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050302 189

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY
(Leave blank)**2. REPORT DATE**
July 2004**3. REPORT TYPE AND DATES COVERED**

Annual Summary (1 Jul 2001 - 30 Jun 2004)

4. TITLE AND SUBTITLEDownstream Signaling Mechanism Underlying MAPK-Induced
Generation of the ER-Negative Phenotype**5. FUNDING NUMBERS**

DAMD17-01-1-0247

6. AUTHOR(S)

Jamie N. Holloway

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)Georgetown University
Washington, DC 20007

E-Mail: hollowaj@georgetown.edu

**8. PERFORMING ORGANIZATION
REPORT NUMBER****9. SPONSORING / MONITORING
AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

Estrogen receptor α (ER α) negative breast tumors often overexpress growth factor receptors, resulting in increased growth factor signaling and hyperactivation of MAPK (ERK1 and ERK2). We have previously shown that ER α -positive MCF-7 cells engineered to stably overexpress various signaling molecules leading to MAPK hyperactivation lose expression of ER α without inducing its transcriptional activation. The downregulation of ER α in these cells is transcriptional and is a specific action of MAPK hyperactivation that is reversible by MAPK abrogation. Here, we show that downregulation of ER α is not mediated specifically by either ERK-1 or -2. TAM67, a construct preventing AP-1 transcriptional activity, was used to determine that AP-1 activity does not play a role in ER downregulation. AP-1 activity is upregulated in response to MAPK activation, and increased AP-1 activity has been observed in ER α negative and hormone independent breast cancers. However, these are the first data indicating mechanistically that despite data correlating increased AP-1 activity with hormone independence/ER α -negativity, increased AP-1 activity is not responsible for ER α downregulation. Use of a dominant negative RSK1 construct indicates that RSK1 activity does not downregulate ER α . Transfection of ERK2 Δ 19-25, which is dominant negative for nuclear MAPK substrates while allowing activation of cytoplasmic substrates, revealed that a cytoplasmic substrate of MAPK is responsible for the generation of the ER α -negative phenotype in these cells. Collectively, these data reveal that the association between increased AP-1 activity and the ER α -negative phenotype is correlative, not causative, and that a cytoplasmic MAPK substrate other than RSK1 is responsible for ER α downregulation in our cell line models.

14. SUBJECT TERMSSignal transduction, MAPK, AP-1, ER α , tamoxifen resistance**15. NUMBER OF PAGES**

27

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	9
References.....	11
Appendices.....	12

Introduction

Upon diagnosis, breast cancer is described as either estrogen receptor (ER)-positive or ER-negative. Patients with ER-positive tumors have a longer disease free and overall survival, and they respond better to hormonal therapies such as tamoxifen, which is easier to tolerate than cytotoxic chemotherapy¹. Conversely, patients with ER-negative tumors tend to have more aggressive disease and must be relegated to much harsher chemotherapy regimens^{2,3}. Unlike ER-positive tumors, ER-negative tumors tend to overexpress growth factor receptors such as EGFR and c-erbB-2, and they have been shown to have high levels of activation of downstream signaling molecules such as MAPK⁴⁻⁶. Previous studies indicated that the hyperactivation of MAPK is directly responsible for the downregulation of ER in breast cancer cells, and that this downregulation is reversible via abrogation of MAPK activity⁷. Consequently, the present study seeks to identify the mechanism of this MAPK induced phenomenon. The outcome of this study has the potential to impact the lives of breast cancer patients who may be able to benefit from a treatment protocol where the blocking of growth factor signaling through MAPK can return ER expression and tamoxifen sensitivity, allowing ER-negative patients to avoid the harsh side effects of cytotoxic chemotherapy.

Body

Statement of Work

Task 1. Identify whether MAPK-induced downregulation of ER α is mediated specifically by ERK1 or ERK2. (months 1-8)

- *Overexpress ERK1 or ERK2 using activated, wild type ERK constructs*
- *Abrogate ERK1 and ERK2 mediated signaling via dominant negative ERK1 and ERK2 constructs*

Addressed in appended manuscript.

Task 2. Identify the role of AP-1 and its composition in ER α downregulation. (months 6-18)

- *Determine AP-1 composition in ER α -negative and ER α + cell lines using fos and jun family member-specific antibodies by Western blotting and antibody supershifting*

Pending final results of the second part outlined in Task 2, this section has not been completed. As preliminary data indicate that abrogation of AP-1 activity does not play a role in the downregulation of ER in these model cell lines, this set of experiments may be omitted. However, Santa Cruz makes a series of antibodies against all fos and jun family members that can be easily obtained should the need arise.

- *Abrogate AP-1 expression using a dominant negative jun construct, Tam67*

Addressed in appended manuscript.

Task 3. Assess the role of cytoplasmic substrates of MAPK in ER α repression. (months 18-36)

- *Determine the localization of the key MAPK substrate*

- Compare $pp90^{RSK}$ activity levels in $ER\alpha$ -negative and $ER\alpha$ + cell lines using anti-phospho- $pp90^{RSK}$ antibody

Addressed in appended manuscript.

- Generation of $pp90^{RSK}$ constructs

A constitutively active RSK construct has been obtained from Dr. Jeffrey Smith, and a dominant negative RSK construct has been obtained from Dr. John Blenis, so the construction of any constructs will not be necessary for the completion of this task.

- Determine if $pp90^{RSK}$ overexpression causes $ER\alpha$ downregulation in $ER\alpha$ + cell lines

Addressed in appended manuscript.

- Determine if AIB1 (activated in breast cancer-1) plays a role in $ER\alpha$ downregulation in $ER\alpha$ + cell lines

Several factors indicated that AIB1 may be a worthwhile target for investigation. In an *in vitro* setting, AIB1 can be phosphorylated by MAPK {684}. In addition, nuclear AIB1 has been shown to have a higher migration when run on a gel next to AIB1 extracted from the cytoplasm {1265}. These data suggest that nuclear AIB1 is phosphorylated, or at least phosphorylated to a greater extent than cytoplasmic AIB1. Taken together, the possibility exists that AIB1 is a cytoplasmic substrate of MAPK. Perhaps AIB1, activated by MAPK phosphorylation, could coactivate the transcription of a repressor of ER transcription, thereby contributing to its downregulation. In addition, AIB1 itself can potentiate NF κ B mediated transcription (246). These data suggest another intriguing scenario in which AIB1 could regulate ER expression. Phosphorylation by MAPK could lead to an increase in active AIB1, which could then coactivate transcription from a promoter containing an NF κ B site, and this increase in transcription may result in ER downregulation. If the proposed model is correct, then AIB1 is a necessary component for the repression of ER, and loss of AIB1 would reverse MAPK-induced ER downregulation. MCF7 cells were transfected with siRNA targeted against AIB1 (a 21-mer corresponding to nucleotides 564-583 of the AIB1 coding region) and an ERE-containing or control (NON) luciferase reporter construct. In addition, the cells were cotransfected with the Δ raf construct or an empty vector. Previous studies showed a consistent downregulation of ER activity in MCF7 cells transfected with the Δ raf construct {739}, and similar results were obtained when cotransfecting control siRNA (figure 1), validating this technique. Figure 1 reveals that there is no difference in estrogen induction of ERE-luciferase in Δ raf containing cells in the presence or absence of AIB1, suggesting that activation of AIB1 does not lead to the downregulation of ER. Interestingly, however, the depletion of AIB1 alone results in a decrease in ERE-luciferase that is similar to the decrease seen with Δ raf transfection. In addition, Δ raf transfection in the absence of AIB1 does not further downregulate ER. These data suggest two possibilities: AIB1 is necessary to coactivate the ER in MCF7 cells, and in its absence fully functional ER can not result in the transcription of ER target genes; or that signaling downstream of raf leads to a decrease in AIB1 protein or activity, and this contributes to ER downregulation.

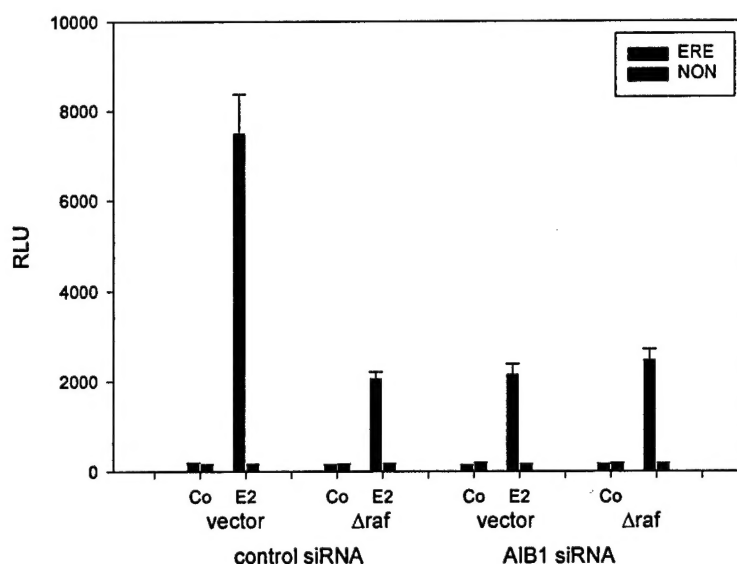


Figure 1. AIB1 depletion results in a decrease in ER activity. MCF7 cells were transfected with 1.25 μ g Δ raf or vector control and 0.75 μ g ERE-luciferase or NON-luciferase as before. In addition, cells were simultaneously transfected with 6 μ L AIB1 or control siRNA. After transfection, the cells were treated with control or estrogen containing medium as indicated and harvested at 48 hours. This is a representative figure of two experiments, each done in triplicate, and the error bars represent s.e.m.

Depletion of AIB1 leads to a decrease in ER protein expression in MCF7 cells. If the downregulation of ER activity seen in the absence of AIB1 is a result of the estrogen receptor's need for AIB1 to fully activate transcription, then the decrease in ER activity should not be correlated with a decrease in ER protein. If, however, there is a decrease in ER protein, then AIB1 may be involved in ER downregulation as suggested in the proposed model above. In fact, ER protein is decreased in MCF7 cells with depleted AIB1; reinforcing the hypothesis that AIB1 may be involved in the downregulation of ER in cells with high MAPK. Densitometric scanning of a western blot of siRNA transfected MCF7 cells reveals that protein expression of AIB1 siRNA reduces ER levels to approximately one-third the levels seen in control siRNA treated cells (Fig. 2). MDA-231 cells are shown as an ER-negative control.

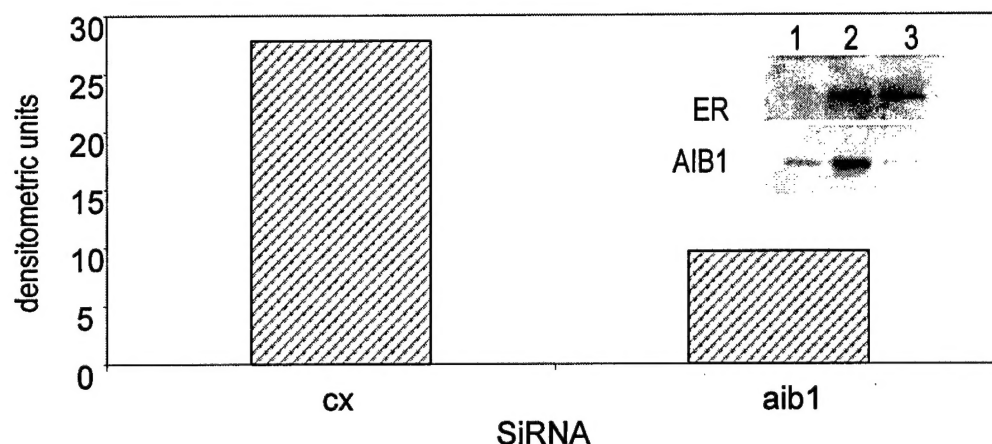


Figure 2. AIB1 depletion results in a decrease in ER protein. MCF7 cells were treated with control (lane 2) or AIB1 (lane 3) siRNA. Cells were harvested at 48 hours, and AIB1 and ER protein levels were assessed by western blotting 30 μ g of whole cell lysates. MDA-231 cells (lane 1) were also included in western blotting as an ER-negative control. Graph represents a densitometric analysis of a representative blot (shown in inset) of two separate experiments.

ER loss and MAPK hyperactivation result in an increase in AIB1 expression. The data thus far seem to correspond with the second proposed model, which is that MAPK activity somehow decreases AIB1 activity or expression, leading to ER downregulation. In this case, cells with MAPK-induced downregulation would be expected to have decreased levels of AIB1 expression or activity. However, both Raf 14c cells and MB3 cells show an increase in AIB1 expression compared to MCF7 cells (Fig 3). Attempts to overexpress Δ raf in MCF7 cells in a transient setting were unfruitful, as the transfection efficiency of MCF7 cells is too low to see any impact of Δ raf transfection on AIB1 expression (data not shown). While it is unlikely that an increase in AIB1 expression is correlated with a decrease in activity, it is at present a very difficult question to address. Currently, the only method for the assessment of AIB1 activity is through the use of reporter constructs containing hormone response elements. However, in this project, it is necessary to assess AIB1 activity independent of any estrogen regulated events, and therefore this analysis cannot be undertaken. Perhaps in the future, the activating phosphorylation sites on AIB1 will be identified, and the development of phospho-specific antibodies will enable the assessment of AIB1 activity independent of an effect on hormone responsive promoters.

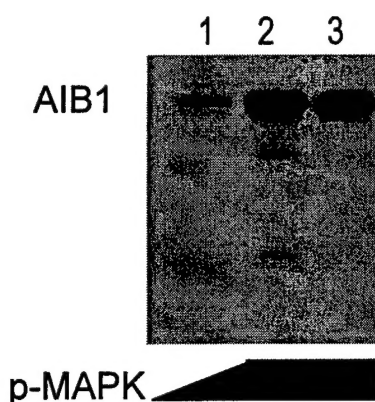


Figure 3. Loss of ER correlates with increased AIB1 expression. MCF7 (lane 1), Raf14c (lane 2), and MB3 (lane 3) cells were grown to approximately 80% confluence. Whole cell lysates were prepared, and western blotting was performed on 30 μ g of each sample. Relative amount of phospho-MAPK are indicated below the figure. This is a representative figure of two experiments.

Like the experiments investigating the role of RSK and AP-1 in ER downregulation, these data indicate that AIB1, as well, does not play a role in MAPK-induced downregulation of ER. Separate pieces of data indicate a role for AIB1 in ER regulation; with AIB1 resulting in the transcription of an ER repressor to downregulate ER (as evidenced by the upregulation of AIB1 in the ER-negative cell lines), or with AIB1 enhancing ER transcription, either directly or through the transcription of a gene coding for a factor which enhances ER transcription (as evidenced by the downregulation of ER protein with AIB1 depletion). However, the data as a whole do not support either conclusion. The fact that AIB1 depletion results in a decrease in ER protein (Fig. 2) is the most compelling data, implicating AIB1 in ER downregulation. However, the summation of the data suggests that any ER downregulation is independent of the ER repression reversed by the abrogation of MAPK signaling.

Key Research Accomplishments

- Determination that the abrogation of either ERK1 or ERK2 or both ERKs in combination leads to the reversal of ER downregulation
- Determination that abrogation of AP-1 mediated transcription does not reverse ER downregulation in ER-negative model cell lines
- Determination that a cytoplasmic substrate of MAPK other than pp90RSK is responsible for the downregulation of ER
- Determination that AIB1 is not responsible for the MAPK-induced downregulation of ER

Reportable Outcomes

Abstracts

Murthy, S., Holloway, J.N., and El-Ashry D. 2004 A cytoplasmic substrate of mitogen activated protein kinase is responsible for estrogen receptor-alpha down-regulation in breast cancer cells: the role of nuclear factor-kappaB. *Mol Endocrinol* 18:1396-1410

Murthy, S., Holloway, J.N., and El-Ashry D. Identification of MAP kinase substrates responsible for the downregulation of ER α in breast cancer cells. 94th Annual Meeting of the American Association for Cancer Research, Washington, DC, 2003, Abstract #1953

Holloway, J.N., Alexander, J., and El-Ashry, D. A Substrate of MAPK is Responsible for the Downregulation of ER α in Breast Cancer Cells. 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, 2002. Abstract # 5332.

Conclusions

Previous data indicated that hyperactivation of MAPK results in the downregulation of ER in ER-positive breast cancer cells, and that this downregulation is reversible through the abrogation of both ERK1 and ERK2, either through MEK inhibition with U0126, or through the use of dominant negative constructs. We have now demonstrated that this ER downregulation is not a result of a specific substrate of either ERK1 or ERK2, as abrogation of either ERK or a combination of the two will result in the return of ER in ER-negative cells. As AP-1 family members are key MAPK substrates, we examined the effect of AP-1 abrogation on ER-negative cell lines to determine if clinical data correlating high AP-1 activity with ER-negativity had a causative relationship. Our data indicate that high AP-1 activity does not result in the downregulation of ER in our model cell lines, and this is the first data demonstrating that while there is significant clinical data correlating ER-negativity with high AP-1 activity, this AP-1 activity is not responsible for the downregulation of ER and acquisition of hormone independence. Experiments with the ERK2 Δ 19-25 construct revealed that the substrate of MAPK responsible for the downregulation of ER resides in the cytoplasm. In addition, use of the dominant negative RSK construct provided data indicating that RSK is not the responsible cytoplasmic substrate. Unfortunately, like AP-1 and RSK, AIB1 is not the MAPK substrate responsible for the MAPK-induced downregulation of ER. Determining the identity of the

MAPK substrate that is responsible for ER downregulation may enable ER-negative patients to be treated with an inhibitor of that specific molecule, returning ER expression and tamoxifen sensitivity, allowing them to be treated with hormonal therapy and forgo the side effects that accompany cytotoxic chemotherapy.

Reference List

1. W. L. McGuire, C. K. Osborne, G. M. Clark, W. A. Knight, *Am.J.Physiol* 243, E99-E102 (1982).
2. W. A. Knight, R. B. Livingston, E. J. Gregory, W. L. McGuire, *Cancer Res.* 37, 4669-4671 (1977).
3. M. Rich, P. Furmanski, S. Brooks, *Cancer Res.* 38, 4296-4298 (1978).
4. M. Toi, A. Osaki, H. Yamada, T. Toge, *Eur.J.Cancer* 27, 977-980 (1991).
5. C. Wright et al., *Br.J.Cancer* 65, 118-121 (1992).
6. V. S. Sivaraman, H. Wang, G. J. Nuovo, C. C. Malbon, *J.Clin.Invest.* 99, 1478-1483 (1997).
7. A. S. Oh et al., *Mol.Endocrinol.* 15, 1344-1359 (2001).

Appendices

“Murthy, S., Holloway, J.N., and El-Ashry D. 2004 A cytoplasmic substrate of mitogen activated protein kinase is responsible for estrogen receptor-alpha down-regulation in breast cancer cells: the role of nuclear factor-kappaB. Mol Endocrinol 18:1396-1410” is appended as file “appendix1.pdf”

A Cytoplasmic Substrate of Mitogen-Activated Protein Kinase Is Responsible for Estrogen Receptor- α Down-Regulation in Breast Cancer Cells: The Role of Nuclear Factor- κ B

JAMIE N. HOLLOWAY, SHALINI MURTHY, AND DORRAYA EL-ASHRY

Lombardi Cancer Center (J.N.H.), Georgetown University, Washington, D.C. 20007; and University of Michigan Comprehensive Cancer Center (S.M., D.E.-A.), Ann Arbor, Michigan 48109-0640

Estrogen receptor α (ER α) negative breast tumors often present with enhanced expression and/or activation of growth factor receptors, resulting in increased growth factor signaling and hyperactivation of MAPK (ERK1 and ERK2). We have previously shown that ER α (+) MCF-7 cells with elevated growth factor signaling lose expression of ER α without any ligand-independent transcriptional activation, and this is a reversible effect attributable to ERK1/2 hyperactivation. Here, we show that down-regulation of ER α is not mediated by a specific ERK-1 vs. ERK-2 substrate. Despite up-regulated activator protein-1 activity in response to ERK1/2 activation, and in ER α (-) and hormone-independent breast cancers, we find that

increased activator protein-1 activity is not responsible for ER α down-regulation. Interestingly, our findings implicate a cytoplasmic substrate of ERK1/2. However, RSK1, the best-characterized cytoplasmic ERK1/2 substrate, does not down-regulate ER α in our models. On the other hand, inhibition of nuclear factor- κ B (which is linked to chemoresistance in cancer in general and has elevated activity in hormone-independent and ER α -breast cancer) significantly enhances ER α activity, suggesting that indirect elevation in nuclear factor- κ B activity (due to hyperactive ERK1/2) is at least partially responsible for ER α down-regulation in these cell line models. (*Molecular Endocrinology* 18: 1396–1410, 2004)

BREAST CANCER CAN present as estrogen receptor α (ER α) positive (+) or negative (-). The presence or absence of ER α is a key prognostic feature of this disease: ER α + tumors have a better prognosis and respond to hormonal therapy (1), whereas ER α - tumors have overall a worse prognosis and are resistant to hormonal therapy (2, 3). ER α + tumors can progress over time and after antiestrogen therapy to ER α - tumors. For example, in patients with ER α + primary tumors who relapse after adjuvant tamoxifen therapy, 50% of the recurrent tumors lack ER α expression. About a third of metastatic tumors that initially respond to tamoxifen subsequently develop resistance and lose ER α expression over this period of time (4, 5). Progression to an ER α - phenotype typically involves the constitutive overexpression of growth promoting genes that are normally regulated

by estrogen, thereby leading to a loss of estrogen dependence, resistance to antiestrogens, and a more aggressive phenotype overall. It is also possible that ER α - tumors develop *de novo*. Immunohistochemical studies of normal breast tissue suggested that approximately 6–12% of the ductal epithelial cells are ER α + (6, 7), although some studies with more sensitive antibodies indicate that the actual number is higher (8). Interestingly, the proliferating ductal epithelial cells in the normal breast do not express ER α (6, 8–11), suggesting that ER α - cells give rise to ER α - tumors. Whether the ER α - phenotype is acquired or *de novo*, the lack of ER α expression denotes a more aggressive phenotype as well as resistance to antiestrogens, and as a result, precludes the use of tamoxifen, relegating patients to more toxic chemotherapies (2, 3).

ER α - tumors and cell lines often overexpress certain growth factor receptors such as the EGFR [epidermal growth factor (EGF) receptor] and c-erbB-2. EGFR and c-erbB-2 overexpression are also important prognostic indicators in breast cancer, independent of their inverse correlation with ER α expression (12–17). This increased growth factor receptor expression and/or activation correlates with increased MAPK activity, both in tumors and in cell lines (12, 18–20). In addition to overexpression and/or constitutive activation of growth factor receptors (with the resulting hyperactivation of MAPK), many hormone-independent

Abbreviations: AP, Activator protein; CCS, charcoal-stripped calf serum; CMV, cytomegalovirus; co-MCF-7, control transfected MCF-7; DMSO, dimethylsulfoxide; dnERK, dominant-negative ERK; dnSK1, dominant-negative RSK1; EGF, epidermal growth factor; EGFR, EGF receptor; ER α , estrogen receptor- α ; ERE, estrogen response element; HB-EGF, heparin-binding EGF; I κ B, inhibitor of κ -B; IKK, I κ B kinase; IMEM, improved MEM; luc, luciferase; MMTV, mouse mammary tumor virus; NF- κ B, nuclear factor- κ B.

Molecular Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

and ER α —tumors also have elevated activator protein (AP)-1 activity (21, 22). Elevated activity of AP-1 and related family members has also been implicated in breast tumor progression in xenograft models: c-jun overexpression in MCF-7 cells results in a hormone-resistant, tumorigenic phenotype (23).

Another transcription factor that might be linked to hormone-independent breast cancer, as well as elevated growth factor signaling, is nuclear factor- κ B (NF- κ B). NF- κ B activity is elevated in hormone-independent breast cancer (24), and is implicated in enhanced cell survival and chemoresistance in cancer (25–27). NF- κ B exists in the cytoplasm in the form of a complex with I κ B (inhibitor of κ -B). Cytokines, chemokines, and intracellular stress lead to the phosphorylation of I κ B by IKK (I κ B kinase), releasing NF- κ B, which can then translocate into the nucleus and modulate the transcription of target genes (reviewed in Ref. 28). Although MAPK does not directly phosphorylate I κ B or activate IKK, there is evidence to show that hyperactivation of MAPK leads to elevated NF- κ B-mediated transcriptional activity through induction of an autocrine factor, most likely heparin-binding EGF (HB-EGF) (29, 30). These data suggest that our model cell lines (with elevated growth factor signaling pathways) might have elevated NF- κ B activity and that this might be involved in the interactions between up-regulated growth factor signaling and decreased ER α expression.

We have previously used stable overexpression of various growth factor signaling components in ER α + MCF-7 breast cancer cells to study the interaction between ER α signaling and growth factor receptor signaling in breast cancer (31–34). Using cell lines that overexpress constitutively active forms of Raf-1, MEK1, or c-erbB-2 and ligand-activatable EGFR, we have shown that the resultant hyperactivation of MAPK (ERK1/2) activity through these signaling pathways leads to the down-regulation of ER α . We have further shown that this down-regulation is not a consequence of ligand-independent ER α activation, and that it is reversible *in vitro*. Abrogation of ERK activity using either pharmacologic inhibitors or dominant-negative ERK (dnERK) constructs reverses the ER α down-regulation and restores ER α activity (34). The objective of this study is to further elucidate the mechanisms by which elevated ERK signaling leads to ER α down-regulation. First, using dnERK1 and dnERK2, we show that the ERK substrate(s) responsible for ER α down-regulation is a common ERK1/ERK2 substrate and not specific to either ERK isoform. Next, the potential role of two key ERK substrates, AP-1 and RSK1, in ER α down-regulation was analyzed. Overexpression and increased activation of AP-1, a transcription factor, has been correlated with hormone independence and the ER α -negative phenotype (21, 35). RSK is a kinase responsible for the activation of various transcription factors (including ER α) and is also involved directly in chromatin remodeling (reviewed in Ref. 36). Although our hyperactive MAPK cell lines

have significantly increased AP-1 activity relative to parental MCF-7 cells, inhibition of this AP-1 activity using a dominant-negative jun does not reverse the ER α down-regulation. Similarly, inhibition of increased RSK activity using a dominant-negative RSK1 (dnRSK1) does not restore ER α expression. Instead, dnRSK1 reduces ligand-induced ER α activation in our control cells, further supporting the possibility that phosphorylation of ER α by RSK enhances ER α transactivation (37). Interestingly, studies with an ERK2 deletion construct that selectively abrogates nuclear ERK1/2 activities show that the ERK substrate responsible for ER α down-regulation is located in the cytoplasm. Finally, we show that these cell lines have elevated NF- κ B activity compared with parental MCF-7 cells, and this elevation in NF- κ B activity is attributable to enhanced growth factor signaling through ERK1/2. Inhibiting this NF- κ B activity either pharmacologically, or through the expression of a constitutively active I κ B [(ca)I κ B], partially restores ER α activity and expression in these cells. Our findings suggest a role for cytoplasmic substrates of MAPK in ER α down-regulation in breast cancer and further support a role for MAPK-induced NF- κ B activity in this down-regulation.

RESULTS

ER α Down-Regulation Due to Hyperactive MAPK Is Not Specifically Attributable to ERK1 or 2

Previous work from our lab has shown that hyperactivation of MAPK results in the down-regulation of ER α protein and message and that this down-regulation is reversible through the abrogation of MAPK (ERK 1/2) signaling, either via pharmacologic inhibition or through expression of dnERK1 and dnERK2 (34). Although ERK1 and ERK2 share many substrates, they have some isoform-specific substrates as well (38). Therefore, our first step in the elucidation of a substrate responsible for ER α down-regulation in breast cancer cells was to determine whether the MAPK-induced down-regulation was a specific effect of the hyperactivation of either ERK1 or ERK2, or whether signaling through either MAPK would down-regulate ER α . Cell line models with drastically reduced ER α expression (described previously in Ref. 34) were obtained by the stable transfection and overexpression of various signal transduction factors into ER α (+) MCF-7 breast cancer cells. These signaling factors were a constitutively active c-Raf-1 [yielding Raf14c cells or (ca)Raf cells] (39), a constitutively active MEK-1 construct (40) [yielding MEK15c or (ca)MEK cells], a wild-type EGFR that can be activated by ligand [MCE5 or EGFR(+) cells] (32), or a wild-type c-erbB-2 [a clone with constitutively high levels of autophosphorylation and constitutive downstream signaling, MB3 or erbB2(+) cells] (33). All of these cell lines grow continuously in the absence of estrogen,

have very high levels of MAPK activity (consistent with the levels of MAPK activity found in ER α - breast cancer cell lines) and express extremely low levels of ER α when compared with control transfected MCF-7 cells (co-MCF7) (34). Each cell line expresses between 4 and 20 fmol of ER α /mg protein, a significant reduction when compared with the control transfected cell lines (Table 1), which exhibit about 120 fmol/mg protein when growing in the continuous presence of estrogen (co-MCF7) or about 400 fmol/mg protein when growing in the continuous absence of estrogen (co-MCF7/s). By selecting single clones of multiple cell lines, all with hyperactive MAPK but generated in different ways, we have substantially reduced the possibility that the reversible down-regulation of ER α seen in these lines is a chance effect due to clonal variation.

To determine whether the ERK substrate responsible for ER α down-regulation is specific to ERK-1 or ERK-2, cells were transiently cotransfected with either an empty vector, dnERK1 (41), dnERK2 (42), or both, in addition to a luciferase (luc) reporter construct (Fig. 1). The estrogen response element (ERE)-luc construct was derived as previously described by the removal of the glucocorticoid response element from the mouse mammary tumor virus (MMTV) promoter. It was replaced with two tandem EREs and then inserted upstream of the luciferase gene. The NON-luc reporter (a critical control that corrects for any nonspecific activation due to the remaining elements of the MMTV promoter) is identical with the ERE-luc, except that the ERE sequences have been scrambled to produce a nonsense sequence. We have previously shown, both by ligand binding assays and by immunohistochemistry, that transfection of a combination of dnERKs 1 and 2 is sufficient to return ER α expression, and through transient transfection assays with ERE-luc and NON-luc that this reexpressed ER α is functional. Importantly, we found that receptor expression, as measured by ligand binding assay or by immunohistochemistry, correlated very well with receptor function as measured by the transient transfection assays with ERE-luc (34), confirming that receptor activity mirrors

receptor expression. In addition to assessing ER expression by ligand binding assay, many studies were performed using either immunohistochemistry or Western blotting (34). Because all of these latter studies used ER α -specific antibodies, and ER α expression measured in this way fully correlated with ER activity by ERE-luc assay, it is presumed that we are looking at ER α -specific effects. Furthermore, the dnERK constructs do not affect ERE-luc activity in the co-MCF7 cells, either in the absence or presence of estrogen (Fig. 1A), again indicating that modulation of ERK activity does not affect the level of activation of preexisting receptor. In all four cell line models with hyperactive MAPK [(ca)Raf in Fig. 1B, (ca)MEK in 1C, erbB2(+) in 1D, and ligand-activated EGFR(+) in 1E], expression of either dnERK is sufficient to restore ER α to levels comparable with the combination of dnERKs 1 and 2 that we previously described as able to return ER α expression. Although any one dnERK alone was perhaps a little less effective at restoring ER α function compared with the combination of both dnERKs, both dnERK-1 and dnERK-2 restored ER α function to levels that would be physiologically relevant. Parallel transfection of the NON-luc does not reveal nonspecific promoter activation, but rather that the ER α activity measured is a direct result of estrogen-induced ER α signaling. Because both dnERKs were sufficient to return ER α to comparable levels, the ERK substrate responsible for ER α down-regulation is unlikely to be specific to either one.

Neither AP-1 nor RSK Are Responsible for ER α Down-Regulation Due to Hyperactive MAPK

AP-1 plays a prominent role in the transcriptional activation of many genes. Furthermore, AP-1 activity is more frequently elevated in ER α - breast cancer (21, 35, 43, 44), and c-jun overexpression confers a hormone-independent, ER α -, tumorigenic phenotype on MCF-7 cells in animal models (23). In addition, the expression of AP-1 family members such as fra-1, fra-2, and some jun family members are specifically induced by MAPK (45, 46). Thus, it would be expected that AP-1 activity would be elevated in systems with hyperactive MAPK. Therefore, to determine the role of elevated AP-1 activity in ER α down-regulation in these MCF-7 models with hyperactive MAPK, a dominant-negative jun construct (TAM67) (47) was used to inhibit AP-1 activity. AP-1 molecules consist of a dimer composed of a jun family member and either another jun family member or a fos family member. TAM67 acts as a dominant negative by forming inactive homo- and heterodimers with jun and fos family members in the cell, thereby eliminating AP-1 mediated transcriptional activity.

The first panel of Fig. 2 represents the transient cotransfection of TAM67 or its parental vector, pCDNA3, along with the AP-1-luc reporter in our model cell lines, as well as in control transfected MCF-7 cells (co-MCF7). All of these cell lines show

Table 1. ER α Expression in MCF-7-Derived Cells with Hyperactive MAPK

Cell Line	ER α Expression (fmol/mg protein)
(ca)Raf	6–10
(ca)MEK	4–7
erbB2(+)	20
EGFR(+) + EGF	6–8
co-MCF7	120
co-MCF7/s	400

ER α expression in our model cell lines was measured by ligand binding assay several times (minimum of three) over a wide range of passage number as described in *Materials and Methods*. The range of receptor levels from these multiple measurements are expressed as femtomoles per milligram of cellular protein.

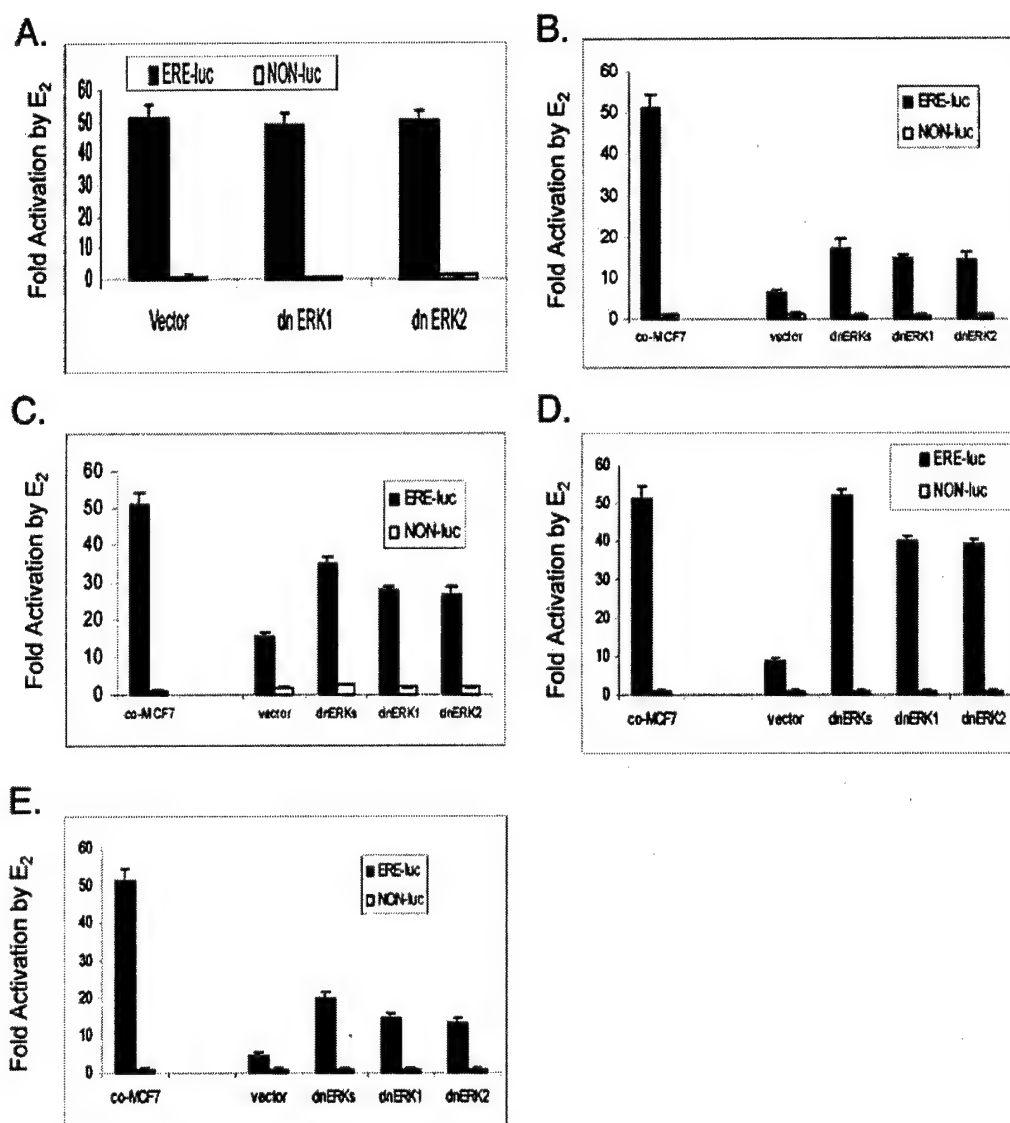


Fig. 1. Down-Regulation of ER α Is Not Mediated Exclusively by Either ERK1 or ERK2

A–E, Co-MCF7, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μ g total dnERK constructs (either dnERK1 alone, or dnERK2 alone, or a 1:1 combination of both dnERKs) and 0.75- μ g luciferase reporter constructs and treated with control or estrogen containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ER α activity. The NON-luc construct is an identical plasmid, except that the EREs are scrambled to result in a nonsense sequence. Experiments are representative of at least three individual experiments, each performed in triplicate. Error bars represent SEM.

elevated AP-1 activity (relative to co-MCF7), which is inhibited 50–75% by the TAM67 construct, reducing the AP-1 activity in these hyperactive MAPK cells to levels comparable to ER α + co-MCF7 cells (Fig. 2A). If AP-1 were playing a central role in the down-regulation of ER α in these breast cancer cells, then the expression of TAM67 (and therefore the specific abrogation of AP-1 activity) in the low ER α model cells should result in the restoration of ER α activity and expression. However, transfection of TAM67 did not restore ER α levels to any significant extent in any of the cell lines (Fig. 2, B–E). In all of these cell lines,

expression of TAM67 did not result in estrogen induction that was significantly different from the vector control, despite AP-1 activity being consistently reduced. Cotransfection of dnERKs, on the other hand, fully restored ER α function in all these cell lines. These data indicate that elevated AP-1 activity does not play a role, either directly or indirectly, in the down-regulation of ER α that results from the hyperactivation of MAPK.

RSK is a cytoplasmic substrate of MAPK that translocates to the nucleus upon activation, activates several transcription factors, and regulates chromatin

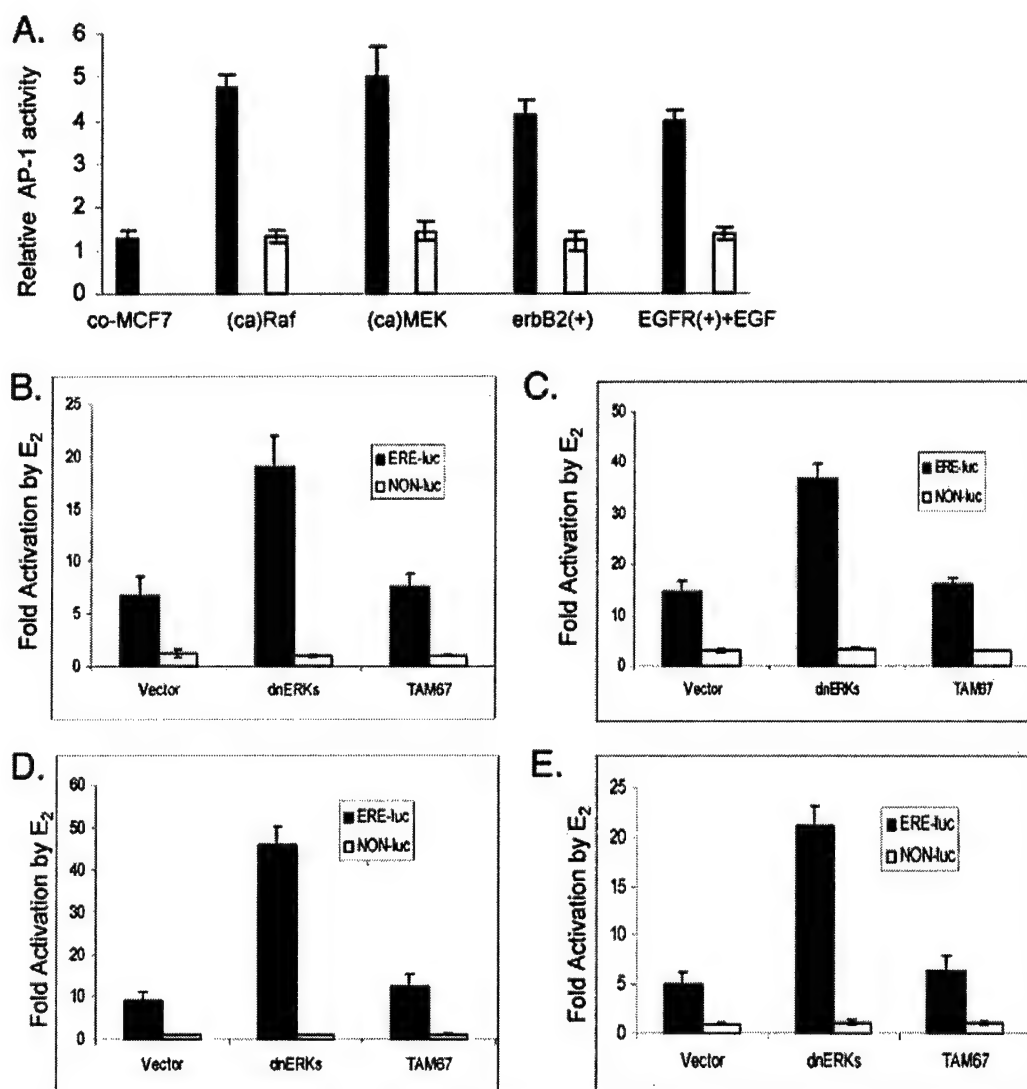


Fig. 2. Down-Regulation of ER α Is Not Mediated by AP-1 Activity

A, Expression of TAM67 inhibits AP-1 activity. Cells were transiently transfected with 1.25 μ g TAM67 or vector control DNA and 0.75 μ g AP-1-luc reporter construct, and basal AP-1 activity was determined. Activity of CMV-luc transfected into the cells in the same experiment was used for normalization and calculation of relative AP-1 activity. The black bars represent basal AP-1-luc activity, whereas the white bars represent AP-1-luc activity upon cotransfection of the TAM67 plasmid. The first bar shows AP-1 activity in co-MCF7 cells. The next four pairs show AP-1 activity in (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells respectively. B–E, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μ g TAM67 (dnjun construct) and 0.75 μ g ERE-luc or NON-luc reporter constructs and were treated with control or estrogen containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ER α activity. Experiments are representative of at least three individual experiments, each performed in triplicate. Error bars represent SEM.

structure through the phosphorylation of histones. Thus, increased activation of RSK1 could lead to induction of a factor that represses ER α expression. On the other hand, RSK1 has also been reported to phosphorylate ER α directly on serine 167, leading to ligand-independent activation of ER α (37). These data indicate that RSK1 might play a role in enhancing ER α activity, either in a ligand-dependent or ligand-independent fashion. In our model cell lines, RSK1 expression is unchanged in response to elevated MAPK ac-

tivity, but its activation correlates with MAPK activity, and therefore is high in these model cell lines (Fig. 3A).

To determine the effects of elevated RSK1 activity on ER α in these model cell lines, a dnRSK1 construct was obtained (48). Because RSK1 had been reported to activate ER α , the effects of dnRSK1 on ER α activity in co-MCF7 cells was first examined. Indeed, cotransfection of dnRSK1 into ER α + co-MCF7 cells results in reduced estrogen-induced transcriptional activation (Fig. 3B), indicating the dnRSK1 has inhibitory activity

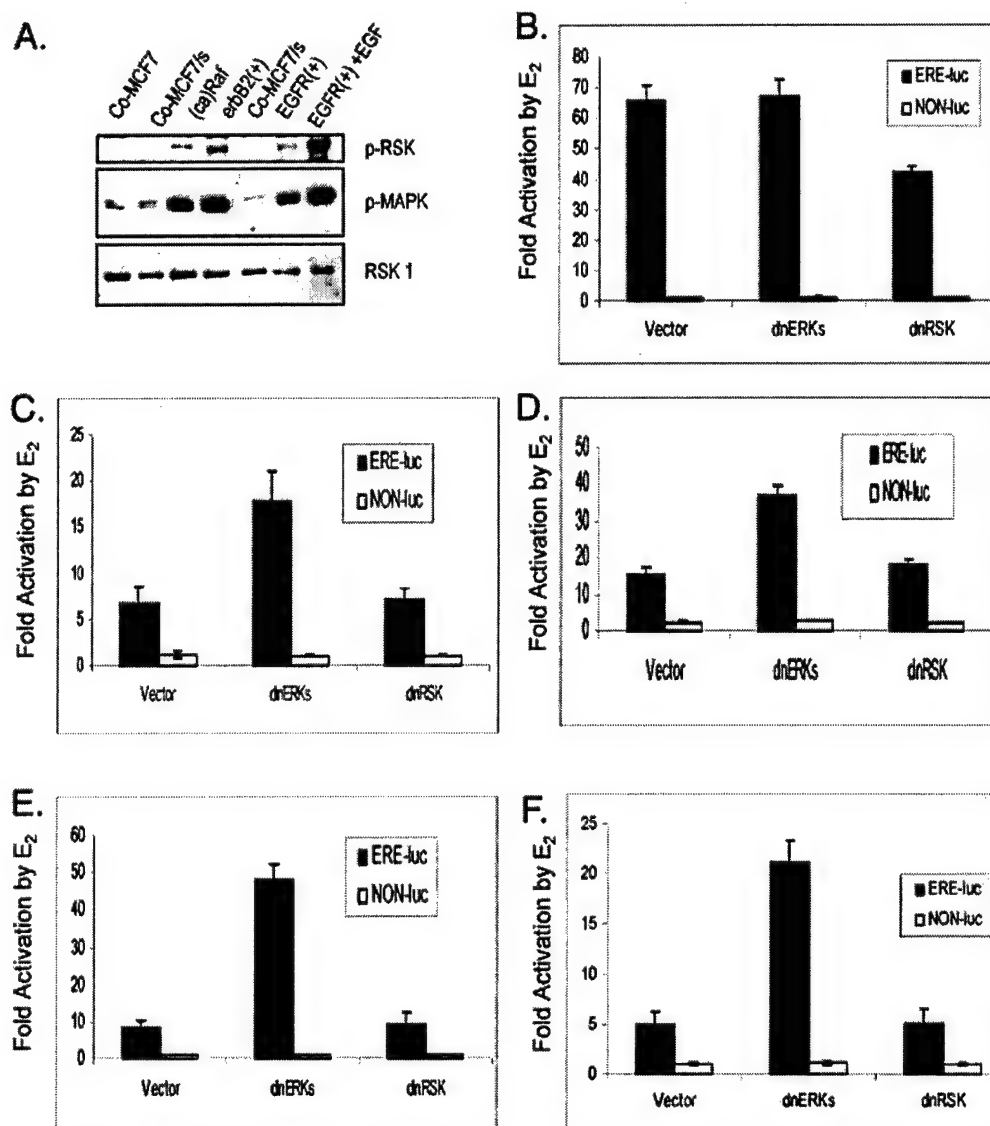


Fig. 3. Down-Regulation of ER α Is Not Mediated by RSK1

A, RSK1 activity correlates with MAPK activity in breast cancer cells. Whole cell lysates were prepared from cells in normal culture conditions grown to approximately 80% confluence. EGFR(+) cells were treated or not with 10 ng/ml EGF for 10 min. Western blots were performed on 5 μ g of total protein for phospho-RSK1 and phospho-MAPK. A Western blot for RSK1 is shown as a loading control. B–F, co-MCF7, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μ g dnRSK1 construct and 0.75 μ g luciferase reporter constructs and were treated with control or estrogen containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ER α activity. Experiments are representative of at least three individual experiments, each performed in triplicate. Error bars represent SEM.

against RSK1. If RSK1 hyperactivation were responsible for the down-regulation of ER α , then expression of dnRSK1 in the low ER α model cell lines would result in the restoration of ER α activity to significantly higher levels (comparable to those seen with dnERKs). However, transient transfection of dnRSK1 into our model cells was not able to restore ER α to levels comparable with dnERK transfection, indicating that RSK1 is not responsible for the MAPK-induced down-regulation of ER α (Fig. 3, C–F). In fact, in co-MCF-7 cells, the estrogen-inducible ER α activity even decreases slightly.

These data collectively indicate that hyperactivation of RSK1 is not mediating the down-regulation of ER α induced by hyperactive MAPK.

A Cytoplasmic Substrate of MAPK Is Responsible for ER α Down-Regulation

MEK acts as a cytoplasmic anchor for MAPK, but upon MEK activation by Raf, MAPK is activated and released into the cytoplasm, where it can activate cytoplasmic substrates such as RSK1. In addition,

active MAPK translocates to the nucleus and phosphorylates nuclear substrates such as elk (reviewed in Ref. 49). A mutant ERK2 construct lacking the region that associates with MEK (ERK2 Δ 19–25) (50) was used to determine the role of cytoplasmic vs. nuclear MAPK activity in ER α down-regulation. Deletion of the MEK association region causes the construct to be constitutively localized in the nucleus, where it cannot be activated by MEK. As a

result, this construct prevents any MAPK activity in the nucleus by binding to (and therefore blocking docking sites on) nuclear MAPK substrates. However, endogenous cellular MAPK can still be activated in the cytoplasm and can continue to activate cytoplasmic substrates. Essentially, this construct functions as a dominant negative in the nucleus without affecting cytoplasmic MAPK signaling (50). As would be expected, nuclear MAPK activity (mea-

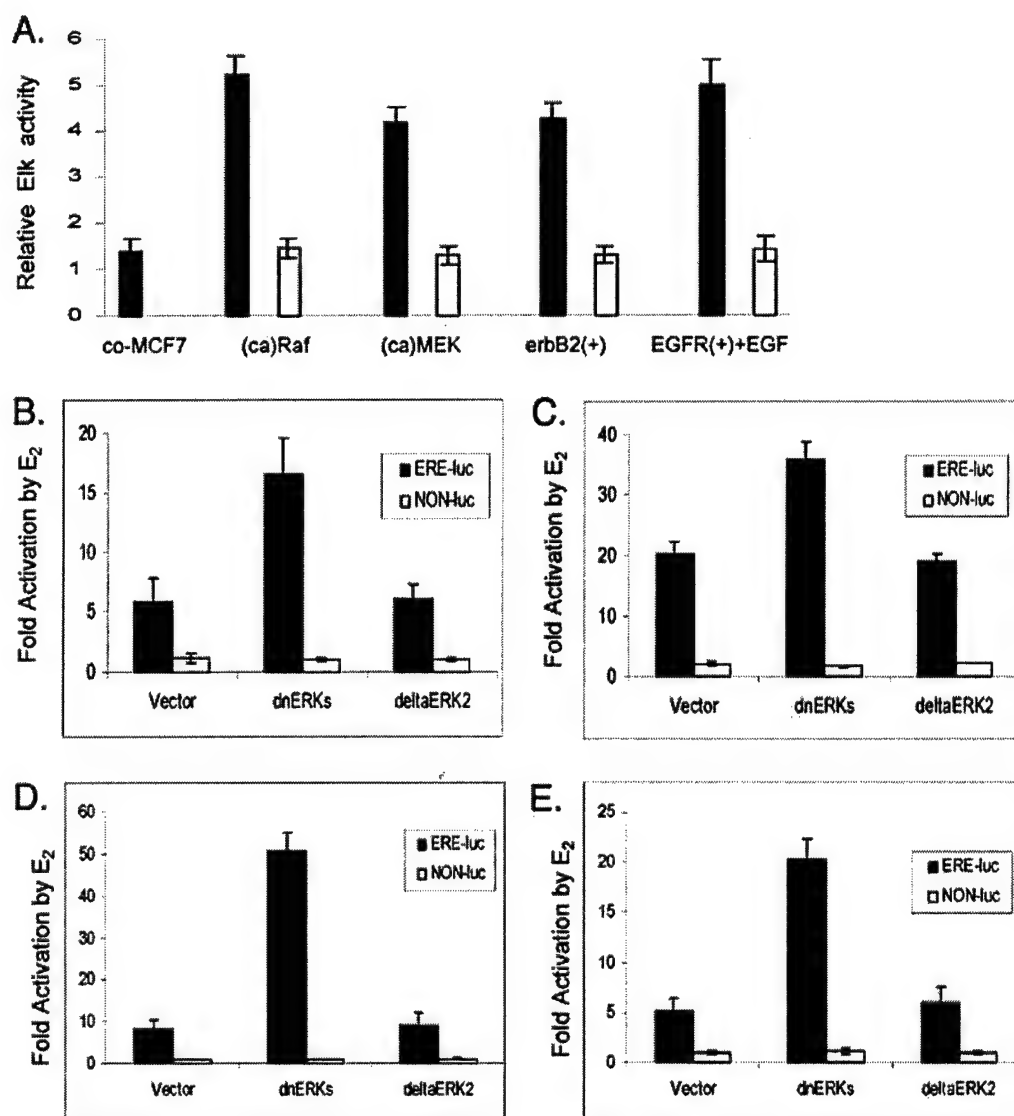


Fig. 4. Down-Regulation of ER α Is Mediated by a Cytoplasmic Substrate of MAPK

A, ERK2 Δ 19–25 represses activity of a nuclear MAPK substrate. Cells were transiently transfected with 0.625 μ g ERK2 Δ 19–25 or vector control DNA, 0.625 μ g pFAelk, and 0.75 μ g pFA-luc reporter construct, and basal Elk activity was determined. Activity of CMV-luc transfected into the cells in the same experiment was used for normalization and calculation of relative Elk activity. The black bars represent basal Elk activity, whereas the white bars represent Elk activity upon cotransfection of the ERK2 Δ 19–25 plasmid. The first bar shows Elk activity in co-MCF-7 cells; the next four pairs show Elk activity in (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells respectively. B–E, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μ g ERK2 Δ 19–25 construct and 0.75 μ g luciferase reporter constructs and were treated with control or estrogen-containing media. Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ER α activity. Experiments are representative of at least three individual experiments, each done in triplicate. Error bars represent SEM.

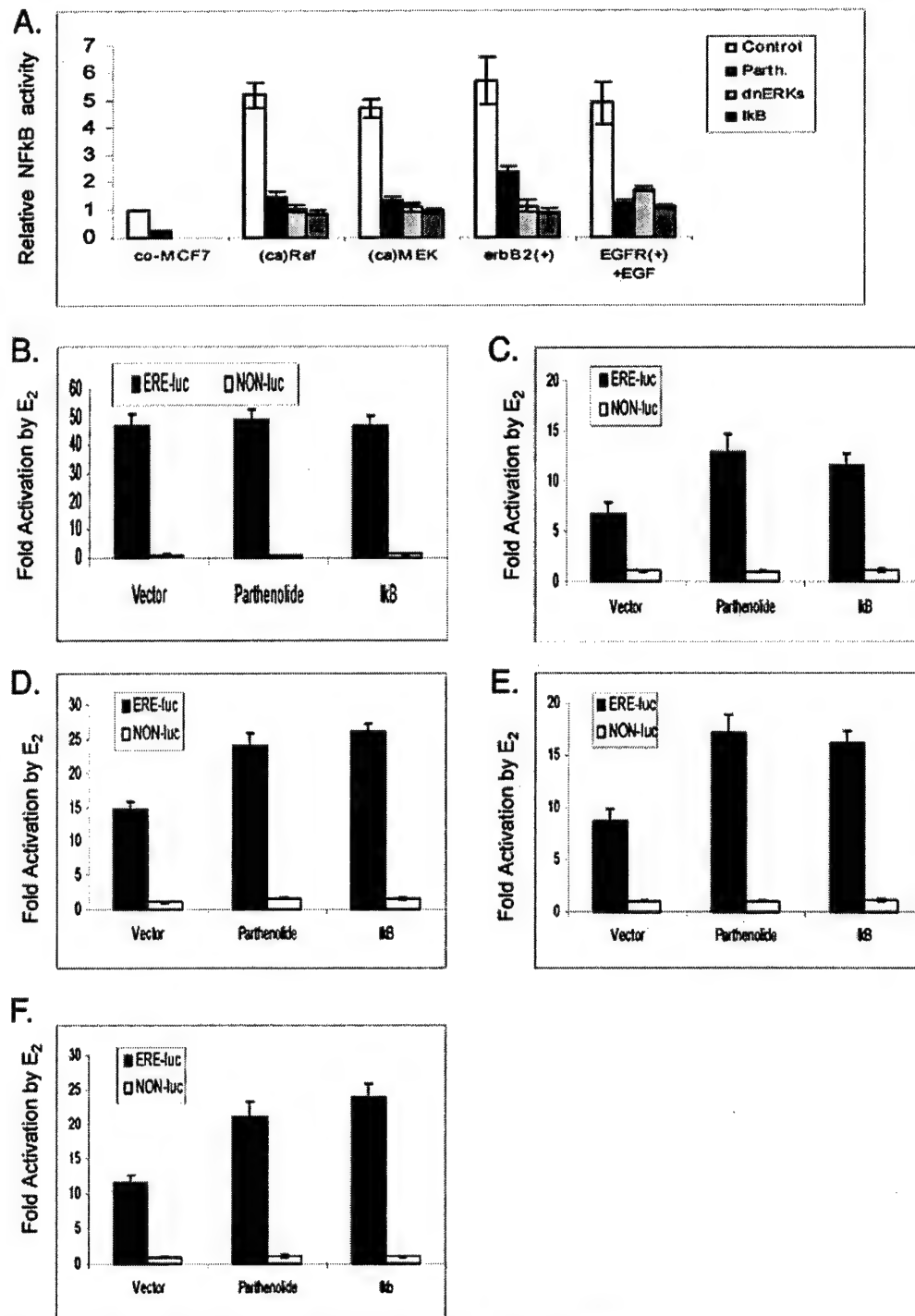


Fig. 5. Elevated NF-κB Activity Is Partially Responsible for ERα Down-Regulation

A, Expression of dnERKs inhibits NF-κB activity. Cells were transiently transfected with 1.25 μg dnERKs, (ca) IκB or vector control DNA and 0.75 μg NF-κB luciferase reporter construct, and basal NF-κB activity was determined. Activity of CMV-luc transfected into the cells in the same experiment was used for normalization and calculation of relative NF-κB activity. The white bars represent basal NF-κB-luc activity, the black bars represent NF-κB-luc activity in the presence of 2 μM Parthenolide, the light gray bars represent NF-κB-luc activity in the presence of dnERKs, and the dark gray bars represent NF-κB activity in the presence of (ca)IκB. B–F, co-MCF7, (ca)Raf, (ca)MEK, erbB2(+), and EGF-treated EGFR(+) cells were transiently cotransfected with 1.25 μg vector DNA or (ca)IκB, and 0.75 μg ERE-luc or NON-luc reporter constructs and were treated with control or estrogen-containing media. One set of plates was treated with 2 μM Parthenolide (DMSO at 1:10,000 was used as vehicle control). Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ERα activity. Experiments are representative of at least three individual experiments, each performed in triplicate. Error bars represent SEM.

sured by Elk activation) is elevated relative to co-MCF-7 in our model cell lines with hyperactive MAPK (Fig. 4A). The ERK2Δ19–25 construct greatly reduces this elevated elk activation to levels comparable to co-MCF-7, *i.e.* it greatly reduces nuclear MAPK activity. If a nuclear substrate were responsible for the down-regulation of ER α , then expression of the ERK2Δ19–25 construct in these ER α -models would be expected to block the activation of that substrate by MAPK, and thus lead to the restoration of ER α activity and expression. However, expression of the ERK2Δ19–25 construct did not lead to the restoration of ER α in any of the cell lines (Fig. 4, B–E); in fact, it was unable to increase ER α activity to above basal levels in all cases and was not able to return ER α activity to the level obtained with dnERK transfection. This indicates that a cytoplasmic substrate of MAPK is responsible for the down-regulation of ER α in these breast cancer cell lines.

The Role of NF- κ B in ER α Down-Regulation by Hyperactive MAPK

NF- κ B activity is elevated in hormone-independent and ER-negative breast tumors (24), and hyperactivation of MAPK leads to enhanced NF- κ B activity through induction of autocrine factors such as HB-EGF (29, 30, 51, 52). We therefore asked whether NF- κ B activity is elevated in MCF-7 breast cancer cells with elevated MAPK activity. As shown in Fig. 5A, NF- κ B activity is about 5-fold higher than parental MCF-7 in all of our model cell lines. This elevated NF- κ B activity is attributable to hyperactivation of MAPK because NF- κ B activity is returned to normal levels (basal levels in co-MCF7 cells) by dnERKs 1 and 2 (Fig. 5A). The elevated NF- κ B activity can also be inhibited by Parthenolide [a specific pharmacological inhibitor of NF- κ B (53)] and through cotransfection of (ca)I κ B (which acts as a dominant negative for NF- κ B, obtained from Upstate Biologicals, Charlottesville, VA). Both (ca)I κ B and Parthenolide, at the usual dose of 2 μ M, result in decreased NF- κ B activity to levels observed in co-MCF7 cells. Neither Parthenolide nor I κ B alter MAPK activity (data not shown); nor does inhibition of NF- κ B activity alter ERE-luc activity in the co-MCF7 cells (Fig. 5B). Interestingly, inhibition of elevated NF- κ B activity in the hyperactive MAPK cell lines with either Parthenolide or (ca)I κ B is about 40–50% as effective in restoring ER α activity as are the dnERKs (Fig. 5, C–F). Increasing doses of Parthenolide do result in further inhibition of NF- κ B activity in the hyperactive MAPK cell lines (Fig. 6A); however, this further decrease in NF- κ B activity does not result in further increases in ER α activity (Fig. 6B), suggesting that the 40–50% effectiveness in restoration of ER α is the maximal effect. Collectively, these data demonstrate that the indirect but specific elevation of NF- κ B activity by hyperactive MAPK plays a role in ER α down-regulation in our cell lines, but it is not the only

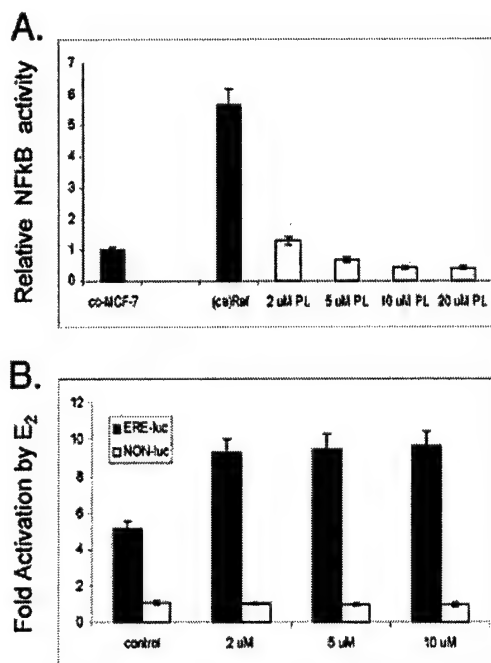


Fig. 6. Elevated NF- κ B Activity Has a Threshold Effect on ER α

A, Higher doses of Parthenolide inhibit NF- κ B activity to levels below basal MCF-7. (ca)Raf cells were transfected with the NF- κ B-luc reporter and luciferase activity was measured in the presence of increasing doses of Parthenolide (2–20 μ M). NF- κ B activity in co-MCF-7 cells was used for comparison. B, Higher doses of Parthenolide have no further effect on NF- κ B activity in cells with hyperactive MAPK. (ca)Raf cells were transiently cotransfected with 1.25 μ g vector DNA and 0.75 μ g ERE-luc or NON-luc reporter constructs and were treated with control or estrogen-containing media. Cells were treated with the indicated doses of Parthenolide (DMSO at 1:10,000 was used as vehicle control). Fold induction in ERE-luc activity after estrogen treatment is used as a measure of ER α activity. Experiments are representative of at least three individual experiments, each done in triplicate. Error bars represent SEM. Similar experiments with the (ca)MEK, erbB-2(+), and EGFR(+) cells yielded similar results (data not shown).

player. Overall, our data suggest that an additional cytoplasmic MAPK substrate(s), along with NF- κ B, leads to ER α down-regulation in MCF-7 cells with hyperactive MAPK.

DISCUSSION

The inverse correlation between ER α and EGFR or c-erbB-2 overexpression/ hyperactivation in breast cancer is well established (54–57). Because ER α -tumors display a more aggressive phenotype, have a poor prognosis, and do not respond to antiestrogen therapy, therapeutic targets enabling the restoration of ER α expression may provide beneficial

treatment strategies. Elucidation of the mechanisms involved in the generation of the ER α - phenotype is crucial for the development of such therapies. We have previously demonstrated that hyperactivation of MAPK induced by enhanced expression and/or activation of EGFR or c-erbB-2 results in down-regulation of ER α expression, suggesting that elevated mitogenic signaling induced by overexpression/ hyperactivation of these growth factor receptors is directly responsible for generating the ER α - phenotype (34). Importantly, abrogation of the hyperactive MAPK restores ER α expression and activity in our model cell lines. To identify the MAPK substrate(s) responsible for down-regulation of ER α expression, the specificity of this response to ERK1 vs. ERK2 was first examined. It has been reported that although ERK1 and 2 share common substrates, they also display some differential substrate specificity (38). In our previous study, we used pharmacologic inhibitors or dnERK1 and dnERK2 combined to inhibit MAPK activity. To determine whether the inhibition of either ERK alone would restore ER α expression/activity, experiments were performed comparing the ability to restore ER α activity by dnERK1 alone or dnERK2 alone relative to the combination of dnERK1 and 2 (Fig. 1). In these experiments, it did not appear that there was a differential ERK substrate involved in down-regulating ER α . However, it is possible that these constructs may not be completely specific, *i.e.* that dnERK1 may inhibit ERK2 to some extent, and *vice versa*. Therefore, to completely rule out a differential ERK specificity, it would be necessary to knock out expression of each ERK separately using small inhibitory RNA or antisense technology.

We next focused on the role of AP-1 in the MAPK-induced down-regulation of ER α expression. Because ERKs induce the expression of various AP-1 family members (45, 58) and result in elevated AP-1 activity, all of our model cell lines displayed increased AP-1 activity. And although AP-1 has been identified as part of a complex that binds to an enhancer element in the ER α promoter (59), up-regulation of AP-1 activity is seen in ER α - and hormone-independent breast cancers (21, 35, 43, 60). In addition, stable overexpression of c-jun in MCF-7 cells leads to the formation of hormone-independent, ER α - tumors in nude mice (23). Collectively, these data seem to implicate elevated AP-1 activity in ER α down-regulation. However, the data presented here (Fig. 2) represent the first mechanistic study to investigate this correlation, analyzing whether inhibition of elevated AP-1 activity consequent to MAPK hyperactivation can restore ER α expression/activity. Results from these experiments show that inhibition of the elevated AP-1 activity in our cell line models is not enough to restore ER α expression, suggesting that MAPK-induced elevation in AP-1 activity does not, on its own, result in the loss of ER α . This is in apparent

contradiction to what might be expected based on the report by Smith *et al.* (23) that overexpression of c-jun leads to the loss of ER α activity and expression in MCF-7 cells. The authors in that report suggest that this might occur either due to squelching of common cofactors required for both ER α -mediated and AP-1-mediated transcriptional activity (similar to that described in Refs. 60 and 61) or maybe through AP-1 activity negatively affecting ER α transcription, although the one AP-1 element identified so far on the ER promoter is a positive regulatory element (59). Because AP-1 molecules can be composed of both homo- and heterodimers, and different dimers have differential affinities for the consensus AP-1 sites, the exact composition of the AP-1 molecule(s) in each instance is likely to influence the final outcome on target genes. It is therefore possible that elevated AP-1 activity due to hyperactive MAPK in our MCF-7-derived cell lines may be due to dimers that markedly differ from those that result from c-jun overexpression, thereby accounting for the very different results reported earlier (23). In addition, AP-1 activity in our hyperactive MAPK cells is three to four times higher than in co-MCF7. Because we don't know the degree to which AP-1 activity is enhanced by c-jun overexpression, it is possible that a threshold level of AP-1 activity is required to alter ER α expression and that this threshold level might be higher than what is seen in our cell lines. Our data here suggests that the negative effects of hyperactive MAPK on ER α transcription are not attributable to MAPK-induced AP-1 but might be due to some other ERK substrate(s), and that inhibiting AP-1 activity is not enough to relieve this effect of hyperactive MAPK.

Data obtained using the ERK2 Δ 19-25 construct (Fig. 4) confirm the fact that AP-1 does not play a role in MAPK-induced ER α down-regulation, as they indicate that nuclear substrates of MAPK, such as the AP-1 family members, are not responsible for ER α down-regulation. In fact, these data point to a cytoplasmic substrate of MAPK as responsible for ER α down-regulation in our model cell lines. Upon activation, this substrate may itself translocate to the nucleus to affect ER α transcription, or it may activate another molecule, either cytoplasmic or nuclear, which results in the loss of ER α .

We have preliminary data indicating that the down-regulation of ER α occurs via both transcriptional repression and induction of protein degradation (our unpublished data). Although these experiments clearly show that the MAPK effect on ER α is not a direct one in the nucleus, that is, interacting with the ER α promoter to result in transcriptional repression, they do not address what role, if any, MAPK may play in any proteasomal degradation of ER α protein that may be occurring. Several reports indicate that MAPK can directly phosphorylate ER α on serine residue 118 (62, 63), and if the phosphorylation of this residue leads to ubiquitination and degradation, then the abrogation of

nuclear MAPK activity could also eliminate this method of down-regulation. However, this effect cannot be identified in a system with transcriptional repression of ER α smaller posttranslational effects on ER α levels would be masked unless the strong transcriptional repression is relieved. Therefore, to elucidate any role of MAPK hyperactivation in ER α degradation, it would be necessary to use tagged ER α constructs under the control of a heterologous promoter.

RSK1 is the best-characterized cytoplasmic substrate of MAPK. However, it is not the cytoplasmic effector that results in ER α down-regulation (Fig. 3). RSK1 is a kinase that is responsible for the activation of many substrates; among them are multiple transcription factors and several histones (reviewed in Ref. 36). RSK1 was a good candidate as the MAPK substrate responsible for the down-regulation of ER α because we had previously established that a major component of ER α down-regulation in our model cells was transcriptional (our unpublished data). The fact that RSK1 itself alters chromatin structure through the phosphorylation of histones, and that many of its substrates are also involved in transcriptional regulation, led us to investigate it initially. In addition, the data provided by the ERK2 Δ 19–25 construct further encouraged us to pursue RSK1 because it is a cytoplasmic substrate of MAPK. Also of interest to us was the fact that RSK1 has been reported to phosphorylate ER α directly, on serine residue 167 (37), although in this case it resulted in ligand-independent activation of ER. Although we show that the activation of RSK1 through MAPK does not lead to ligand-independent activation of ER α , as the phosphorylation and results from (37) might suggest, it appears that RSK1 may play a role in the ligand-dependent activation of ER α . Abrogation of signaling through RSK1 did not restore estrogen-induced ER activity, and in fact, it seemed to decrease the ability of estrogen to induce ER α activity, at least in the control (MCF-7) cells. This implicates the phosphorylation of ER α on S167 as potentially important in traditional estrogen-dependent signaling. This result also confirms that the dnRSK1 construct is functional and has an effect on cell signaling. Therefore, a cytoplasmic substrate of MAPK other than RSK1 must be responsible for the down-regulation of ER α induced by hyperactivation of MAPK in these breast cancer cells.

It has been known for some time that NF- κ B activity is elevated in hormone-independent and ER α -breast cancers (24). However, the correlation between NF- κ B activity and MAPK was less clear—it is now evident that, whereas MAPK does not directly activate NF- κ B (either through I κ B phosphorylation, or through activation of IKK), hyperactive MAPK enhances NF- κ B activity through induction of an autocrine factor, likely HB-EGF (30, 51, 52). Thus, hyperactivation of MAPK can indirectly lead to elevated NF- κ B activity. We have shown here that

our MCF-7-derived model cell lines all have high basal levels of NF- κ B activity as a consequence of ERK1/2 hyperactivation (Fig. 5). Interestingly, inhibiting this level of NF- κ B activity (bringing it down to levels normally seen in MCF-7 cells) partially restores ER α activity in these cell lines, indicating that loss of ER α is attributable to elevated NF- κ B activity, along with (other) cytoplasmic substrate/s of ERK1/2. It is important to note that, under our experimental conditions, ERE-luc activity mirrors ER α expression [as we have shown earlier (34)], indicating that increase in ER α activity after NF- κ B inhibition is attributable to restoration of ER α expression. This is further reinforced by the fact that NF- κ B inhibitors have no effect on ERE-luc activity in co-MCF7 cells. Therefore, the enhanced ERE-luc activity in our hyperactive MAPK cell lines after NF- κ B inhibition is due to increased ER α expression, rather than due to increased activation of preexisting receptor through modulation of coactivators and/or corepressors. We also find that further reducing NF- κ B activity beyond the basal level inherent to MCF-7s does not increase ER α activity any further (Fig. 6), suggesting that down-regulation of ER α due to deregulation of NF- κ B activity is a threshold effect. The basal level of activity seen in MCF-7 cells has no adverse effect on ER α ; rather, it is the elevation in NF- κ B activity beyond this limiting level, which contributes to down-regulating ER α expression. This threshold effect mimics that observed for hyperactivation of MAPK; that is, we had previously observed that neither the basal MAPK activity levels in MCF-7 cells nor the modest elevation of MAPK that occurs in estrogen-independent MCF-7 cells (those long-term adapted for growth in the absence of estrogen such as our co-MCF-7/s cells) has a detrimental effect on ER α expression levels; only hyperactivation to a very high level as seen in ER α -breast cancer cells resulted in down-regulation of ER α expression (34).

This and future studies further elucidating the interaction/s between hyperactive growth factor signaling and ER α expression in breast cancer could have substantial clinical impact in patients. Methylation of the ER α promoter is present at the time of diagnosis of breast cancer in about 25% of ER α -breast cancer cases (64), and in these tumors abrogation of MAPK activity would not be expected to return ER α expression (because demethylation of the ER α promoter would have to occur first). However, in patients with ER α -tumors without any hypermethylation of the promoter, a therapeutic regimen that combines a signal transduction inhibitor/a monoclonal antibody against a key signaling molecule, along with an antiestrogen, could prove beneficial. Iressa, which inhibits the kinase activity of EGFR, and Herceptin, a monoclonal antibody raised against c-erbB-2, can both be expected to reduce MAPK activity, and would be good candidates for such combination therapies. Although further stud-

ies are essential to show that these inhibitors of mitogenic signaling can restore ER α expression/function in breast tumors, coupling signal transduction inhibitors or monoclonal antibodies with antiestrogens could result in therapies that are better tolerated than standard chemotherapy for the treatment of ER α - breast cancer. In addition, once the MAPK substrate/s responsible for ER α down-regulation is identified, that could provide an additional drug target. The implication of elevated NF- κ B activity in development of the ER α - phenotype also has clinical potential. High NF- κ B activity is thought to contribute to resistance to chemotherapy and to radiation-induced apoptosis in cancers generally (25–27), as well as to influence tumor growth and metastasis through promoting cell survival and cell cycle entry (27, 65). Interestingly, it has been reported that cotreatment with NF- κ B inhibitors enhances the paclitaxel sensitivity of MDA-MB-231 breast cancer cells (which have very high basal NF- κ B activity) (66). Therefore, combinations of NF- κ B inhibitors with standard chemotherapy/radiation might prove generally beneficial in breast and other cancers. Added to this, our data (establishing a role for NF- κ B in the loss of ER α expression) make NF- κ B a very attractive candidate for combination therapies in breast cancer.

MATERIALS AND METHODS

Cell Culture and Reagents

All cells with hyperactive MAPK were maintained in phenol red-free improved MEM (IMEM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% charcoal-stripped calf serum (CCS). Control transfected MCF-7 cells were maintained in IMEM supplemented with 10% fetal bovine serum. All cell lines used in this study have been described earlier (34); the (ca)MEK cells were generated by transfection of MCF-7 cells as described before (34, 39) using a constitutively active MEK construct [a generous gift from Dr. Natalie Ahn (40)]. After transfection, cells were grown in PRF-IMEM/10% CCS supplemented with 10 μ g/ml gentamicin (Invitrogen Life Technologies). Hormone treatments after transfections were performed by supplementing the gentamicin containing CCS media with 17 β -estradiol (Sigma, St. Louis, MO), at a final concentration of 10⁻⁹ M; in the case of EGF-treated EGFR(+) cells, the medium was also supplemented with 10 ng/ml EGF (Upstate Biologicals). Parthenolide was obtained from Sigma, and stored as a 10,000 \times stock in dimethylsulfoxide (DMSO) at -20 C. Lipofectamine and PLUS reagent were obtained from Invitrogen. Cells were seeded in 75-cm² T-flasks (Costar, Cambridge, MA) and grown in a forced air humidified incubator at an atmosphere of 5% CO₂ and 37 C.

Plasmids

Luciferase reporter plasmids (pGLB-mERE and pGLB-mNON) were obtained by the insertion of an altered MMTV promoter containing a tandem repeat of a consensus ERE or a scrambled version of the same sequence instead of the glucocorticoid response element into the HindIII site of the pGLB basic plasmid (Promega Corp., Madison, WI) (67).

The dnERK1 and dnERK2 constructs and parental vector pCep4L were kindly provided by Dr. Melanie Cobb (University of Texas Southwestern, Dallas, TX). The TAM67 construct was a gift of Dr. Powel Brown (Baylor College of Medicine Breast Center, Houston, TX). The ERK2 Δ 19–25 construct was a gift of Dr. Michael Weber (University of Virginia, Charlottesville, VA), and Dr. John Blenis (Harvard Medical School, Boston, MA) kindly provided the dnRSK1 and pRK7 (parental vector) constructs. pCep4L (dnERK parental vector), pCDNA3 (TAM67 and ERK2 Δ 19–25 parental vector), and pRK7 (dnRSK1 parental vector) were used as empty vector controls in transient transfections. The Stratagene PathDetect kit was the source of the AP-1-luc, NF- κ B-luc, pFAelk, and pFRluciferase plasmids. The constitutively active I κ B plasmid [(ca)I κ B or I κ B (S32A/S36A) in pUSE] was obtained from Upstate Biologicals.

Transfections

Cells were plated at approximately 60–70% confluence in Falcon six-well plates and allowed to attach overnight. They were then transfected using the LipoPlus method. Briefly, 100 μ l serum-free IMEM per well was mixed with 4 μ l Plus reagent and 2 μ g total plasmid DNA [0.75 μ g of each luciferase containing reporter plasmid, and 1.25 μ g total of the effector plasmid(s)]. This mixture was incubated at room temperature for 15 min, combined with a mixture containing 100 μ l serum-free IMEM and 4 μ l Lipofectamine, and then incubated for an additional 15 min. This mixture was then added to the cells, which were incubated for 3 h at 5% CO₂ and 37 C. After transfection, the cells were washed with PBS and incubated for 48 h in appropriate treatment media. Control transfection with a cytomegalovirus (CMV)-luc plasmid was performed in all experiments as a measure of transfection efficiency. All transfections in each experiment were performed in triplicate.

Luciferase Assays

Cells were washed twice with PBS and lysed in 200 μ l of passive lysis buffer (Promega) with gentle agitation for 15 min at room temperature. Ten to 25 μ l of lysate (actual volumes determined based on the amount needed to get CMV-luc readings in the middle of the linear range of the instrument) were used to measure luciferase activity using Promega's Luciferase Assay system. The luciferase values (relative light units) were normalized for protein concentration. Values were corrected by the subtraction of relative light units per microgram of protein for the mock-transfected cells. Triplicates were averaged, and fold activation by estrogen was plotted; the error bars represent SEM. Results shown are representative of at least three experiments with similar results, each done in triplicate.

Ligand Binding Assays

Whole cell extracts were prepared from cells grown to 75–80% confluence as described before (34). Extracts were diluted to a concentration of 2 mg/ml, and incubated with 10 nM [³H]-17 β -estradiol in the presence and absence of \times 100 unlabeled estradiol for 16 h at 4 C. Dextran-coated charcoal was then used to adsorb free hormone and pelleted by centrifugation. Aliquots of the supernatant were counted in 10 ml of liquid scintillation fluid using a Beckman Coulter Inc. (Fullerton, CA) liquid scintillation counter. Receptor expression values were determined as described in Ref. 68 and are expressed as femtomoles per milligram of protein. Assays were performed a minimum of three times on each cell line over time in culture.

Acknowledgments

We acknowledge Drs. Sofia Merajver, Francis Kern, and James Rae for critical review of this manuscript and thoughtful and helpful discussions.

Received February 5, 2004. Accepted March 22, 2004.

Address all correspondence and requests for reprints to: Dorraya El-Ashry, University of Michigan Comprehensive Cancer Center, 1150 West Medical Center Drive, MSRB III, Room 5220B/5323, Ann Arbor, Michigan 48109-0640. E-mail: elashryd@umich.edu.

This work was supported by Department of Defense Grant DAMD17-01-0247 (to J.N.H.); American Cancer Society Grant RSG-01-0330-01 (to D.E.A.); Susan G. Komen Foundation Grant BCTR2000565 (to D.E.A.); and National Institutes of Health-University of Michigan Cancer Center Support Grant 5 P30 CA46592.

J.N.H. and S.M. made equal contributions.

REFERENCES

- McGuire WL, Osborne CK, Clark GM, Knight WA 1982 Steroid hormone receptors and carcinoma of the breast. *Am J Physiol* 243:E99-E102
- Knight WA, Livingston RB, Gregory EJ, McGuire WL 1977 Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res* 37:4669-4671
- Rich M, Furmanski P, Brooks S 1978 Prognostic value of estrogen receptor determinations in patients with breast cancer. *Cancer Res* 38:4296-4298
- Johnston SR, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, Ebbs SR, Dowsett M 1995 Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res* 55:3331-3338
- Newby JC, Johnston SR, Smith IE, Dowsett M 1997 Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer. *Clin Cancer Res* 3:1643-1651
- Anderson E, Clarke RB, Howell A 1998 Estrogen responsiveness and control of normal human breast proliferation. *J Mammary Gland Biol Neoplasia* 3:23-35
- Petersen OW, Hoyer PE, Van Deurs B 1987 Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. *Cancer Res* 47:5748-5751
- Clarke RB, Howell A, Potten CS, Anderson E 1997 Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res* 57:4987-4991
- Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP, Sibson DR, Sloane JP 1999 Estrogen receptor-positive proliferating cells in the normal and precancerous breast. *Am J Pathol* 155:1811-1815
- Ballare C, Bravo AI, Laucella S, Sorin I, Cerdeiro R, Loza J, Sousa MF, Guman N, Mordoh J 1989 DNA synthesis in estrogen receptor-positive human breast cancer takes place preferentially in estrogen receptor-negative cells. *Cancer* 64:842-848
- Russo J, Ao X, Grill C, Russo IH 1999 Pattern of distribution of cells positive for estrogen receptor α and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Res Treat* 53:217-227
- Gee JM, Robertson JF, Ellis IO, Nicholson RI 2001 Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. *Int J Cancer* 95:247-254
- Nicholson S, Richard J, Sainsbury C, Halcrow P, Kelly P, Angus B, Wright C, Henry J, Farndon JR, Harris AL 1991 Epidermal growth factor receptor (EGFR); results of a 6 year follow-up study in operable breast cancer with emphasis on the node negative subgroup. *Br J Cancer* 63:146-150
- Toi M, Hamada Y, Nakamura T, Mukaida H, Suehiro S, Wada T, Toge T, Niimoto M, Hattori T 1989 Immunocytochemical and biochemical analysis of epidermal growth factor receptor expression in human breast cancer tissues: relationship to estrogen receptor and lymphatic invasion. *Int J Cancer* 43:220-225
- Hainsworth PJ, Henderson MA, Stillwell RG, Bennett RC 1991 Comparison of EGFR, c-erbB-2 product and ras p21 immunohistochemistry as prognostic markers in primary breast cancer. *Eur J Surg Oncol* 17:9-15
- Lewis S, Locker A, Todd JH, Bell JA, Nicholson R, Elston CW, Blamey RW, Ellis IO 1990 Expression of epidermal growth factor receptor in breast carcinoma. *J Clin Pathol* 43:385-389
- Toi M, Nakamura T, Mukaida H, Wada T, Osaki A, Yamada H, Toge T, Niimoto M, Hattori T 1990 Relationship between epidermal growth factor receptor status and various prognostic factors in human breast cancer. *Cancer* 65:1980-1984
- Jeng MH, Yue W, Eischeid A, Wang JP, Santen RJ 2000 Role of MAP kinase in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. *Breast Cancer Res Treat* 62:167-175
- Hermanto U, Zong CS, Wang LH 2000 Inhibition of mitogen-activated protein kinase kinase selectively inhibits cell proliferation in human breast cancer cells displaying enhanced insulin-like growth factor I-mediated mitogen-activated protein kinase activation. *Cell Growth Differ* 11:655-664
- Seddighzadeh M, Zhou JN, Kronenwett U, Shoshan MC, Auer G, Sten-Linder M, Wiman B, Linder S 1999 ERK signalling in metastatic human MDA-MB-231 breast carcinoma cells is adapted to obtain high urokinase expression and rapid cell proliferation. *Clin Exp Metastasis* 17:649-654
- Dumont JA, Bitonti AJ, Wallace CD, Baumann RJ, Cashman EA, Cross-Doersen DE 1996 Progression of MCF-7 breast cancer cells to antiestrogen-resistant phenotype is accompanied by elevated levels of AP-1 DNA-binding activity. *Cell Growth Differ* 7:351-359
- Johnston SR, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M, Benz CC 1999 Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. *Clin Cancer Res* 5:251-256
- Smith LM, Wise SC, Hendricks DT, Sabichi AL, Bos T, Reddy P, Brown PH, Birrer MJ 1999 cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. *Oncogene* 18:6063-6070
- Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet Jr RJ, Sledge Jr GW 1997 Constitutive activation of NF- κ B during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 17:3629-3639
- Liu ZG, Hsu H, Goeddel DV, Karin M 1996 Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF- κ B activation prevents cell death. *Cell* 87:565-576
- Higgins KA, Perez JR, Coleman TA, Dorshkind K, McCormas WA, Sarmiento UM, Rosen CA, Narayanan R 1993 Antisense inhibition of the p65 subunit of NF- κ B blocks tumorigenicity and causes tumor regression. *Proc Natl Acad Sci USA* 90:9901-9905

27. Wang CY, Mayo MW, Baldwin Jr AS 1996 TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF- κ B. *Science* 274:784–787
28. Siebenlist U, Franzoso G, Brown K 1994 Structure, regulation and function of NF- κ B. *Annu Rev Cell Biol* 10: 405–455
29. Norris JL, Baldwin Jr AS 1999 Oncogenic Ras enhances NF- κ B transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. *J Biol Chem* 274: 13841–13846
30. Pearson G, English JM, White MA, Cobb MH 2001 ERK5 and ERK2 cooperate to regulate NF- κ B and cell transformation. *J Biol Chem* 276:7927–7931
31. Osborne CK, Jarman M, McCague R, Coronado EB, Hilsenbeck SG, Wakeling AE 1994 The importance of tamoxifen metabolism in tamoxifen-stimulated breast tumor growth. *Cancer Chemother Pharmacol* 34:89–95
32. Miller DL, El-Ashry D, Cheville AL, Liu Y, McLeskey SW, Kern FG 1994 Emergence of MCF-7 cells overexpressing a transfected epidermal growth factor receptor (EGFR) under estrogen-depleted conditions: evidence for a role of EGFR in breast cancer growth and progression. *Cell Growth Differ* 5:1263–1274
33. Liu Y, El-Ashry D, Chen D, Ding IYF, Kern FG 1995 MCF-7 breast cancer cells overexpressing transfected *c-erbB-2* have an *in vitro* growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity *in vivo*. *Breast Cancer Res Treat* 34:97–117
34. Oh AS, Lorant LA, Holloway JN, Miller DL, Kern FG, El-Ashry D 2001 Hyperactivation of MAPK induces loss of ER α expression in breast cancer cells. *Mol Endocrinol* 15:1344–1359
35. Daschner PJ, Ciolino HP, Plouzek CA, Yeh GC 1999 Increased AP-1 activity in drug resistant human breast cancer MCF-7 cells. *Breast Cancer Res Treat* 53: 229–240
36. Frodin M, Gammeltoft S 1999 Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol* 151:65–77
37. Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J, Lannigan DA 1998 pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol Cell Biol* 18:1978–1984
38. Chuang CF, Ng SY 1994 Functional divergence of the MAP kinase pathway. ERK1 and ERK2 activate specific transcription factors. *FEBS Lett* 346:229–234
39. El-Ashry D, Miller DL, Kharbanda S, Lippman ME, Kern FG 1996 Constitutive Raf-1 kinase activity in breast cancer cells induces both estrogen-independent growth and apoptosis. *Oncogene* 15:423–435
40. Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG 1994 Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265:966–970
41. Kortenjann M, Shaw PE 1995 Raf-1 kinase and ERK2 uncoupled from mitogenic signals in rat fibroblasts. *Oncogene* 11:2105–2112
42. Robinson MJ, Harkins PC, Zhang J, Baer R, Haycock JW, Cobb MH, Goldsmith EJ 1996 Mutation of position 52 in ERK2 creates a nonproductive binding mode for adenosine 5'-triphosphate. *Biochemistry* 35: 5641–5646
43. Gee JM, Ellis IO, Robertson JF, Willsher P, McClelland RA, Hewitt KN, Blamey RW, Nicholson RI 1995 Immunocytochemical localization of Fos protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. *Int J Cancer* 64:269–273
44. Bamberger AM, Methner C, Lisboa BW, Stadtler C, Schulte HM, Loning T, Milde-Langosch K 1999 Expression pattern of the AP-1 family in breast cancer: association of fosB expression with a well-differentiated, receptor-positive tumor phenotype. *Int J Cancer* 84:533–538
45. Treinies I, Paterson HF, Hooper S, Wilson R, Marshall CJ 1999 Activated MEK stimulates expression of AP-1 components independently of phosphatidylinositol 3-kinase (PI3-kinase) but requires a PI3-kinase signal to stimulate DNA synthesis. *Mol Cell Biol* 19:321–329
46. Gee JM, Barroso AF, Ellis IO, Robertson JF, Nicholson RI 2000 Biological and clinical associations of c-jun activation in human breast cancer. *Int J Cancer* 89:177–186
47. Ludes-Meyers JH, Liu Y, Munoz-Medellin D, Hilsenbeck SG, Brown PH 2001 AP-1 blockade inhibits the growth of normal and malignant breast cells. *Oncogene* 20: 2771–2780
48. Roux PP, Richards SA, Blenis J 2003 Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. *Mol Cell Biol* 23:4796–4804
49. Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K, Cobb MH 2001 Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22:153–183
50. Eblen ST, Catling AD, Assanah MC, Weber MJ 2001 Biochemical and biological functions of the N-terminal, noncatalytic domain of extracellular signal-regulated kinase 2. *Mol Cell Biol* 21:249–259
51. McCarthy SA, Samuels ML, Pritchard CA, Abraham JA, McMahon M 1995 Rapid induction of heparin-binding epidermal growth factor/diphtheria toxin receptor expression by Raf and Ras oncogenes. *Genes Dev* 9:1953–1964
52. Troppmair J, Hartkamp J, Rapp UR 1998 Activation of NF- κ B by oncogenic Raf in HEK 293 cells occurs through autocrine recruitment of the stress kinase cascade. *Oncogene* 17:685–690
53. Koo TH, Lee JH, Park YJ, Hong YS, Kim HS, Kim KW, Lee JJ 2001 A sesquiterpene lactone, costunolide, from *Magnolia grandiflora* inhibits NF- κ B by targeting I κ B phosphorylation. *Planta Med* 67:103–107
54. Toi M, Osaki A, Yamada H, Toge T 1991 Epidermal growth factor receptor expression as a prognostic indicator in breast cancer. *Eur J Cancer* 27:977–980
55. Wright C, Nicholson S, Angus B, Sainsbury J, Farndon JR, Cairns J, Harris AL, Horne CHW 1992 Relationship between *c-erbB-2* protein product expression and response to endocrine therapy in advanced breast cancer. *Br J Cancer* 65:118–121
56. Sivaraman VS, Wang H, Nuovo GJ, Malbon CC 1997 Hyperexpression of mitogen-activated protein kinase in human breast cancer. *J Clin Invest* 99:1478–1483
57. Sharma AK, Horgan K, Douglas-Jones A, McClelland R, Gee J, Nicholson R 1994 Dual immunocytochemical analysis of oestrogen and epidermal growth factor receptors in human breast cancer. *Br J Cancer* 69: 1032–1037
58. Murakami M, Ui MIH 1999 Fra-2-positive autoregulatory loop triggered by mitogen-activated-protein kinase (MAPK) and Fra-2 phosphorylation sites by MAPK. *Cell Growth Differ* 10:333–342
59. Tang Z, Trilleux I, Brown M 1997 A transcriptional enhancer required for the differential expression of the human estrogen receptor in breast cancers. *Mol Cell Biol* 17:1274–1280
60. Doucas V, Spyrou G, Yaniv M 1991 Unregulated expression of c-Jun or c-Fos proteins but not Jun D inhibits oestrogen receptor activity in human breast cancer derived cells. *EMBO J* 10:2237–2245
61. Tzukerman M, Zhang XK, Pfahl M 1991 Inhibition of estrogen receptor activity by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate: a molecular analysis. *Mol Endocrinol* 5:1983–1992

62. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270:1491–1494
63. Smith CL 1998 Cross-talk between peptide growth factor and estrogen receptor signaling. *Biol Reprod* 58:627–632
64. Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, Baylin SB, Issa J-PJ, Davidson NE 1996 Methylation of estrogen and progesterone receptor genes 5' CpG islands correlates with ER and PR gene expression in breast tumors. *Clin Cancer Res* 2:805–810
65. Mayo MW, Wang CY, Cogswell PC, Rogers-Graham KS, Lowe SW, Der CJ, Baldwin Jr AS 1997 Requirement of NF- κ B activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* 278:1812–1815
66. Patel NM, Nozaki S, Shortle NH, Bhat-Nakshatri P, Newton TR, Rice S, Gelfanov V, Boswell SH, Goulet Jr RJ, Sledge Jr GW, Nakshatri H 2000 Paclitaxel sensitivity of breast cancer cells with constitutively active NF- κ B is enhanced by I κ B α super-repressor and parthenolide. *Oncogene* 19:4159–4169
67. El-Ashry D, Lippman ME, Kern FG, Human transforming growth factor- α contains an estrogen responsive element comprised of two imperfect palindromes. *Proc 73rd Annual Meeting of The Endocrine Society*, Washington, DC, 1991, p 268 (Abstract 950)
68. Edwards DP, McGuire WL 1980 17 α -Estradiol is a biologically active estrogen in human breast cancer cells in tissue culture. *Endocrinology* 107:884–891



Molecular Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.